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14. ABSTRACT Dr. Truica previously showed that the cell adhesion molecule β -catenin forms a complex with the androgen receptor (AR) and modulate its transcription. The cross talk between β -catenin and AR signaling can play an important role in AR transcriptional in prostate cancer progression. Our preliminary data seem indicate stromally derived paracrine Wnt family members activate the epithelial frizzled receptor to enable prostate epithelial survival in an androgen deficient environment. We will continue to test the original hypothesis that there is a direct molecular interaction between β -catenin and the Cterminus region of AR involved in the mechanism of prostate androgen responsiveness. However, we will examine the repercussions of the interaction in both LNCaP (originally proposed) and primary cultures of mouse prostatic stromal cells. The physiologic response to androgen ablation (castration) differ significantly between the prostatic stroma and epithelia despite the common expression of β -catenin and AR, as evidence for the different transcriptional cofactor interactions found in prostatic epithelial and stromal cells. The future work will adhere to the previously approved tasks and those detailed in the body of this report with the additional examination of prostatic stromal cells.					
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INTRODUCTION

The mortality due to prostate cancer primarily results from the failure of androgen-ablation therapy and metastasis to distant sites. Prostate cancer becomes androgen independent as a result of documented mutations in androgen receptor (AR, 50%) and unknown factors (50%) (1-3). New treatments are required that are more effective irrespective of the structure of the AR in the cancerous epithelial cells. Previously the oncogene β -catenin has been shown to be mutated in human prostate cancer (4, 5). β -Catenin has a role in cell-cell adhesion and is an essential signaling molecule in the Wnt signaling pathway where it acts as a transcriptional coactivator for the T cell factor (TCF) family of proteins (6, 7). The canonical Wnt signaling is associated with elevated proliferation and neoplastic transformation of various epithelial tissues (8). The cross talk between β -catenin and AR signaling can play a role in prostate cancer progression (9-12). The original objective of this proposal was to determine the mechanism by which β -catenin modulates AR signaling. However due to the recent intensity of research in this field and research delays from transferring the grant from Dr. Cristina Truica (original PI) to the current PI, the proposal outlook has been broadened.

Reported observations of the PI show that a mouse model lacking transforming growth factor beta (TGF- β) signaling in fibroblasts that spontaneously develop pre-neoplastic lesions in the prostate (13). More recent unpublished data suggest that the prostate tissue the same mouse model is insensitive to androgen ablation and has altered β -catenin expression compared to normal mice. Thus the cross-talk among the β -catenin, androgen signaling, and TGF- β signaling pathways have a potentially important role as a whole in prostate androgen responsiveness. Further, the mouse model suggests, the prostatic stromal fibroblasts are critical to epithelial androgen signaling. Dr. Truica had found that the β -catenin, with a complex of other proteins that she has identified, could interact with the androgen receptor and effect DNA binding. She has also began to examine the role of androgen ablation on these protein-DNA interactions. The tools that she has developed in the past two years of funding can be used to answer important questions in the molecular interactions involved in the mechanism androgen responsiveness of the prostate. We will continue to test the original hypothesis that there is a direct molecular interaction between β -catenin and the C-terminus region of AR involved in the mechanism of prostate androgen responsiveness. However, we will examine the repercussions of the interaction in both LNCaP (originally proposed) and primary cultures of mouse prostatic stromal cells. The physiologic response to androgen ablation (castration) differ significantly between the prostatic stroma and epithelia despite the common expression of β -catenin and AR, as evidence for the different transcriptional cofactor interactions found in prostatic epithelial and stromal cells.

BODY

The approved statement of work comprised of three main tasks:

Task 1. Investigate the effects of loss-of-function of β -catenin on the LNCaP prostate epithelial cells

Task 2. Mechanisms involved in the development of androgen independent prostate cancer.

Task 3. Determine if E-cadherin and AR compete to binding the same domain of β -Catenin.

We have made progress toward these goals in the past seven months of undertaking the grant with an additional emphasis on prostatic stromal cells. As reasoning for the added examination of the prostatic stromal cells as stated above, we have done castration experiments in transgenic mice deficient in TGF β signaling in stromal cells of fibroblast origin (Figure 1). To conditionally knockout the TGF β type II receptor in the fibroblastic compartment, mice having loxP sites at introns 1 and 2 of *Tgfr2* were crossed with mice expressing Cre recombinase under the control of the FSP1 (fibroblast specific protein-1; S100A4) promoter. FSP1 is selectively expressed in fibroblasts (14, 15) during embryologic development after E8.5 (16). The F β KO male mice demonstrated a consistent fibroblastic hyperplasia in the seminal vesicles and the dorso-lateral, ventral, and anterior prostate lobes. The stromal phenotype was accompanied by epithelial hyperplasia having hyperchromatic nuclei with atypia similar to human PIN in each of the prostate lobes in 100% of the male mice examined (13). These observations highlight the importance of TGF β signaling in stromal fibroblasts and how T β RII ablation in the stroma can mediate epithelial transformation in trans. For the purposes of this project the role stromal TGF- β signaling has on prostatic epithelial androgen responsiveness is pertinent. Specific experiments are being done to examine the TGF- β signaling pathway in prostatic stromal cells regulating stromal differentiation and prostate cancer progression with support from another DOD grant W81XWH-04-01-0046. The studies in this grant will specifically focus on the β -catenin and androgen receptor pathway in the prostatic epithelia and stromal cells.

The interactions between AR and β -catenin promote AR-transcriptional activity in LNCaP cells (17). Previously described experiments (last progress report) AR and β -catenin bind the PSA promoter at a similar time scale upon dihydro-testosterone (DHT) treatment. Further anti-androgen, bicalutamide, treatment inhibits AR and β -catenin loading of the PSA enhancer element, but has little effect on the promoter, ARE1 (androgen response element 1) based on ChIP analysis. These studies were successfully repeated in the PI's laboratory upon taking over the grant. The additional question remained, whether AR and β -catenin interact at the site of transcription in vivo. This could only be answered through the technique of sequential ChIP analysis. This was done by first immunoprecipitating AR and performing PCR to verify ARE3 interaction. Then the primary antibody was disassociated, and the immunoprecipitate was re-immunoprecipitated

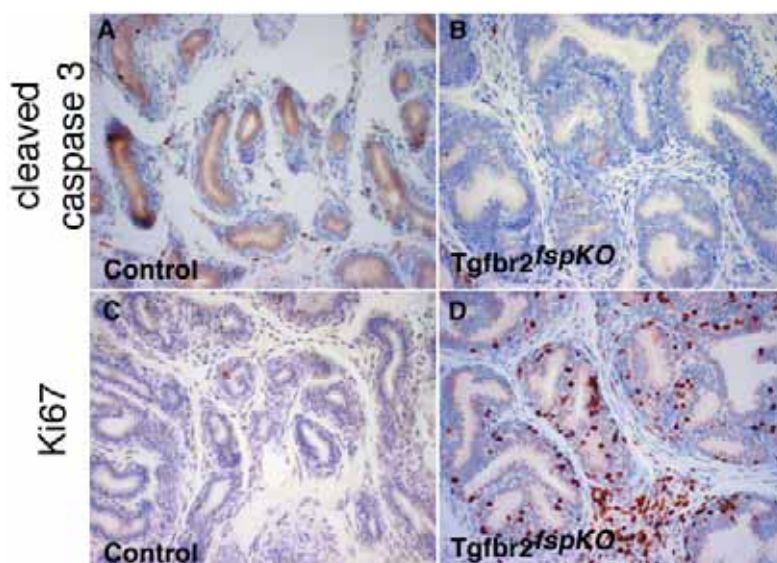


Figure 1. Prostates were examined four days following castration. The prostates were stained for (B, C) cleaved-caspase3, a early marker for apoptosis., or (D, E) Ki-67 expression, for proliferation. Accordingly, there is elevated apoptosis and decreased proliferation in control mice compared to *Tgfr2^{fspko}* after castration.

with a second antibody, for β -catenin (Figure 2). Transcriptional regulation of β -catenin/TCF response element by AR was then tested using luciferase assay. Examination of β -catenin transcriptional activity on the TCF binding site show that AR has little effect alone, but both DHT and bicalutamide could inhibit TCF transcriptional activity (Figure 3). Independent of AR-specific transcriptional activities the regulation of β -catenin signaling through by

Figure 2. ChIP and the AR-DHT or AR-bicalutamide complex suggest another mechanism by which AR-independent prostate cancer differ AR-dependent the PSA enhancer prostate tissue. As evidence, the mutation of the C-terminal AR element, AR loads position, V716R, was unable to suppress TCF transcriptional activity on ARE3 within 1h through β -catenin binding (Figure 3). Further studies will be done in of DHT incubation in both LNCaP and prostatic stromal cells for β -catenin mutations S33Y LNCaP cells. Re-ChIP (constitutively active) and deletion of armadillo repeat 6 (disables AR analysis shows that β -catenin binds to the AR interactions will be determined to test whether E-cadherin and AR immunoprecipitated compete for β -catenin binding [Task 3]. The implications are not only cell lysate. important to our understanding androgen responsiveness of metastatic prostate cancer often deficient in E-cadherin expression, but also the differences between epithelial and stromal androgen responsiveness since stromal cells do not express E-cadherin.

In response to castration or androgen ablation, the prostatic stromal regression is one third of the epithelial response, although both express β -catenin and AR (18). Primary prostatic stromal fibroblasts were grown from 8 week old mouse prostates by previously reported methods (19). Interestingly, the cultured cells maintained

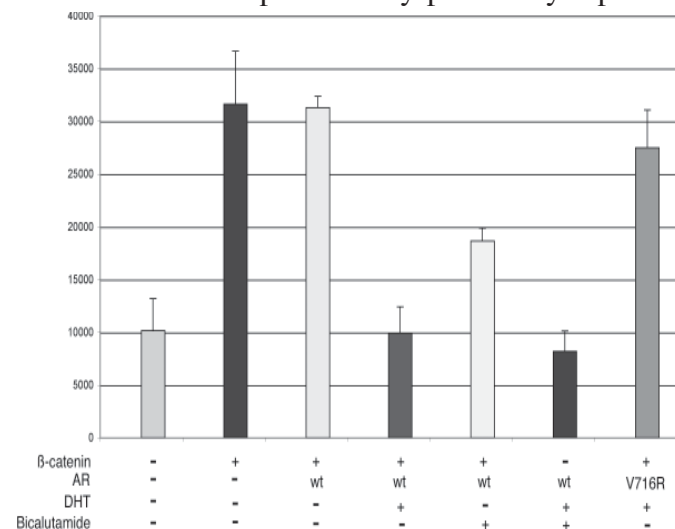


Figure 3. Luciferase reporter assay for TCF transcriptional activity. AR suppresses β -catenin-mediated activation of the reporter in the presence of DHT or bicalutamide. However, AR mutant Z716R has little effect on β -catenin trans activation.

smooth muscle differentiation as in the intact mice, as determined by positive co-immunofluorescence staining for α -smooth muscle actin (α -SMA) and desmin (Figure 4A). No vimentin expression was observed. Further staining for AR in the cultured stromal cells treated with DHT (1 h) indicated continued AR expression (Figure 4B); this was important since many cultured prostatic epithelial cells lose AR expression (20-22). Further experiments showed that β -catenin and AR co-immunoprecipitate upon DHT treatment (1 h) but do not associate in the absence of DHT (Figure 4C)

In an effort to understand the role of prostatic epithelial androgen responsiveness [Task 2], we examined the expression of various secreted proteins by stromal cells. Based on the lack of androgen responsiveness in the stromal conditional Tgfrb2 knockout and the importance of the β -catenin pathway on AR transcriptional regulation. We focused on the expression of the Wnt family of genes

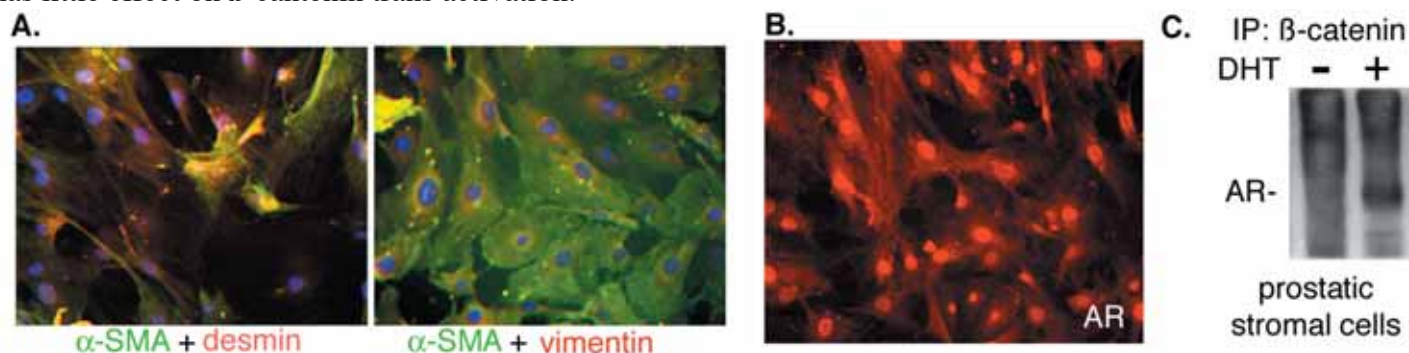


Figure 4. Mouse prostatic stromal cells exhibit similar interaction of AR and β -catenin in the presence of DHT. (A) Coimmunofluorescence for α -smooth muscle actin (α -SMA, green) and desmin (red) displays a yellow fluorescence, suggestive of smooth muscle differentiation. The lack of vimentin expression (red) support the differentiation assessment. (B) The stromal cells express AR in the nucleus 1 h following DHT treatment. (C) β -catenin immunoprecipitate with AR in the presence of DHT (1 h) as determined by Western blotting.

from the stromal cells of the *Tgfr2* conditional knock out and control mice. The cells were incubated with bicalutamide for 24 –96h or ethanol (vehicle). Due to the lack of availability of good quality antibodies for many of proteins in this family, the RNA expression was measured through realtime RT-PCR. Interestingly, we found that expression levels of *Wnt2* to be elevated in the *Tgfr2* knockout stromal cells with *Wnt9a* and *Wnt11* suppressed compared to wild type control (Figure 5). The expression level of *Wnt4*, *Wnt5a*, and *Wnt5b* were similar in both *Tgfr2* knockout and control stromal cells. All realtime RT-PCR reactions were done in triplicate with corresponding standard curve for quantitation of each gene and GAPDH for loading control. The differential expression of *Wnt* genes in an androgen non-responsive prostate mouse model lends support to our studies of the β -catenin/AR interactions in the prostate epithelia. *Wnt2* activates the canonical β -catenin/TCF signaling pathway associated with promotion of proliferation, whereas *Wnt9a* and *Wnt11* stimulate the non-canonical downstream signaling associated with growth suppression, morphogenesis, and apoptosis (23-25). The fact that these *Wnt* family members are differentially regulated also support a complex a relationship between the stroma and epithelia as well as the novel identification of androgen dependent *Wnt* signaling activation.

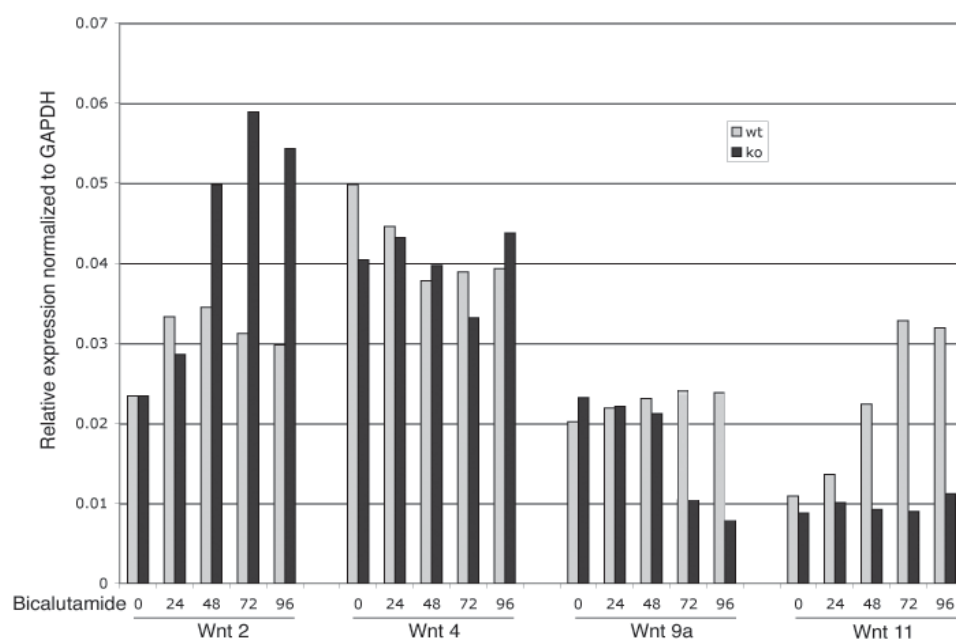


Figure 5. Expression of *Wnt* genes are differentially androgen regulated in prostatic stromal cells. Stromal cells from control (wt) and *Tgfr2* knockout (ko) cells were incubated with bicalutamide for 0-96 h. the RNA was extracted and RT-PCR performed for *Wnt 2*, *Wnt 4*, *Wnt9a*, and *Wnt 11*. There was an observable increase in *Wnt 2* expression in the ko cells with androgen depletion, whereas *Wnt4* expression was similar in both cell types. *Wnt 9a* was suppressed by bicalutamide treatment in the ko cells relative to wt, but *Wnt 11* was elevated in the wt cells over basal levels under similar conditions.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrate AR can suppress β -catenin signaling in the presence of DHT.
- Showed that the dynamic loading of β -catenin and AR through re-ChIP experiments on the PSA enhancer and promoter regions.
- Prostatic stromal cells exhibit AR and β -catenin interactions similar to prostatic epithelial cells.
- Prostatic stromal cells from androgen-nonresponsive prostate tissue have differential expression of Wnt genes compared to control prostatic stromal cells.
- We identified that Wnt11 and Wnt 9a are androgen-regulated genes.

CONCLUSIONS AND FUTURE WORK

The data accumulated thus far from Dr. Truica's work and those by the PI's laboratory is sufficiently mature that is being put together for manuscript submission currently. The future work will adhere to the previously approved tasks and those detailed in the body of this report with the additional examination of prostatic stromal cells. Thus further support is being requested to do complete the studies on the role of androgen and β -catenin signaling involved in androgen responsiveness of the prostatic epithelia.

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